Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Diagnostic value of cfDNA and long fragment DNA in patients with breast cancer

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Received May 28, 2020; Accepted October 20, 2020; Published October 31, 2020

Doi: http://dx.doi.org/10.14715/cmb/2020.66.7.28

Abstract: To investigate the diagnostic value of cell-free DNA (cfDNA) and long fragment DNA in breast cancer patients.Female patients with breast cancer (n = 80) were recruited over one year for this study, and served as an observation group. The control group consisted of 50 normal, healthy females. Plasma levels of cfDNA and long fragment DNA were determined a day before treatment, 7 days after treatment, and on the 20th day of treatment. The levels of cfDNA and long fragment DNA in breast cancer patients before treatment were significantly higher than those of the control group (p<0.05). Patients cfDNA and long fragment DNA levels 7 days after treatment were not significantly different from the corresponding values at 1 day before treatment (p>0.05), but they decreased significantly on the 20th day of treatment, when compared with levels before treatment (p<0.05). Before treatment, the optimal cut-off point for cfDNA in patients' peripheral blood, sensitivity, specificity and accuracy were 12.25ng/mL, 79.12%, 86.15%, and 73.32%, respectively. The area under the ROC curve (AUC) was 0.865 (95% CI = 0.754-0.903). Close monitoring of cfDNA levels in peripheral blood of breast cancer patients in real-time can be used for early diagnosis of the disease.

Key words: Breast cancer; Cell-free DNA; Diagnosis; Long fragment DNA; Plasma.

Introduction

Breast cancer, the most common malignancy in women, accounts for 25.1% of all cancer cases worldwide, and it seriously affects the quality of life of women. The incidence of breast cancer is higher in developed countries, while relative mortality is higher in less developed countries. In all countries, the education of women has been suggested for the early detection and treatment of breast cancer. Strategies for the control and prevention of breast cancer must be of high priority to health policymakers, and it is also necessary to increase awareness of risk factors and early detection in less developed countries (1, 2). Epidemiological studies have revealed that about 1.2 million women come down with breast cancer annually, and the incidence keeps rising by about 5 to 20% (3). Advancements in medical technology have impacted positively on the diagnosis and treatment of breast cancer (4). Although this has greatly reduced mortality from breast cancer, the disease remains the leading cause of death in women (4). Early diagnosis and treatment are key to the effective management of the disease. This has necessitated the search for novel biomarkers that can effectively diagnose breast cancer and offer a good prognosis.

Strategies currently employed to treat breast cancer require that a patient first undergoes an imaging examination based on clinical manifestations presented. The insidious nature of breast cancer makes it difficult for early diagnosis (5). There is presently a dearth of tumor markers for early diagnosis of the disease (6). Plasma cfDNA is derived mainly from apoptosis and necrosis of cells. Its expression level reflects various pathological and physiological processes. Studies have shown that the level of cfDNA is significantly higher in tumor patients than in normal healthy individuals, and it is mostly due to tumor cell necrosis and secretion (7, 8). Studies have also shown that cfDNA fragments that originate from tumor cells are shorter than those that originate from non-malignant cells. It was recently demonstrated that the size-selection of smaller cfDNA fragments could be used to increase the amount of tumor-derived cfDNA fragments in cfDNA samples. Long fragment DNA is a representative test index of cfDNA, which reflects changes in tumor burden of patients (9, 10). The present study investigated the diagnostic value of cfDNA and long fragment DNA in breast cancer patients.

Materials and Methods

Materials

Human genomic DNA was a product of Beijing Huake Jianlian Gene Technology Co. Ltd. Tris-EDTA buffer was obtained from Shanghai Jingke Chemical Technology Co. Ltd., while TGL-20 type centrifuge was purchased from Qingdao Mingbo Environmental Technology Co. Ltd.

Patients and general information

Female patients with breast cancer (n = 80) were recruited over one year for this study and served as an observation group. There were 62 cases of invasive ductal carcinoma of the breast, 4 cases of invasive lobular carcinoma, 2 cases of invasive mucinous carcinoma, 2 cases of infiltrating medullary carcinoma, and 10 cases of mixed breast cancers. According to the degree of

Table 1. Primer sequences used for qRT-PCR.

Primer		Sequence
Primer 1	Forward	5'-TG-GCACATATACACCATGGAA-3'
	Reverse	5'-TGAGAATGATTTTCCAATTT-3'
Primer 2	Forward	5'-ACACCTATTCCAAAATTGACCAC-3'
	Reverse	5'-TTCCCTCTACACACTGCTTTGA-3'

tumor differentiation, there were 31 cases of low differentiated adenocarcinoma, and 49 cases of high/moderately differentiated adenocarcinoma. There were 42 cases with tumors >2cm, and 31 cases with lymphatic metastasis. The control group consisted of 50 normal healthy females. The included patients signed written informed consent with their family members. There were no significant differences in their clinicopathological characteristics. The study protocol was approved by the Clinical Research Ethics Committee of our hospital.

Inclusion and exclusion criteria

The included subjects were: (1) patients diagnosed with primary breast cancer via pathological examination; (2) patients who had not received chemotherapy, anti-tumor drugs and other related treatments; (3) patients without dehydration, acute inflammation and trauma; and (4) patients who together with their family members signed informed consent. The excluded patients were: (1) those with infectious diseases, (2) pregnant or lactating women, (3) patients with other malignant tumors, (4) patients with autoimmune deficiency diseases; and (5) patients with blood system diseases.

Blood sample collection and preparation of plasma

Patients' fasting peripheral venous blood (10mL) was collected in the early morning in EDTA anticoagulant bottles 1 day before treatment, 7 days after treatment and on the 20th day of treatment. The blood samples were centrifuged at 16, 000 rpm for 10 min to obtain plasma. Aliquots of the plasma (200μL) were immediately used for DNA extraction or refrigerated at -80°C before use.

Determination of plasma cfDNA and long fragment DNA levels

The plasma was thawed on ice and spun at 10,000g for 3 min before DNA purification. The extracted DNA was eluted with a 50μL Tris-EDTA elution buffer using the QIAamp DNA Blood Mini Kit. The purified DNA was then quantified or refrigerated at -20°C. A serial dilution of a standardized solution of human genomic DNA was used for the preparation of a standard calibration curve, and the levels of cfDNA and long fragment DNA in each sample were extrapolated from the standard curve.

qRT-PCR

The qRT-PCR reaction was performed in triplicate. The reaction mixture (20 μL) consisted of 1μL DNA template, 0.5μL each of forward and reverse primer (LINE1 97 or LINE1 259), 10 μL UltraSYBR Mixture, and 8μL double-distilled water. The cycling conditions were 1 min at 95°C, and 35 cycles of 95°C for 8 sec, and 60°C for 15 sec. Each plate consisted of a serum DNA sample, a negative control (distilled water) and 7 serially diluted standard DNA solutions (11). The primer sequences used for the qRT-PCR are shown in Table 1.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS (21.0). Groups were compared using Student's *t*-test and chi-squared test. Statistical significance was assumed at $p<0.05$.

Results

Comparison of patients' clinical data

There were no significant differences in age and BMI between the two groups ($p > 0.05$; Table 2).

Levels of cfDNA in peripheral blood of patients and controls

The level of cfDNA in breast cancer patients before treatment was significantly higher than that of the control group ($p<0.05$). Patients' cfDNA level 7 days after treatment was not significantly different from the corresponding level at 1 day before treatment ($p>0.05$) but decreased significantly on the 20th day of treatment when compared with the corresponding level before

Table 2. Comparison of clinical data of the two groups.

Clinical data	Group		
	Observation (n = 80)	Control (n = 50)	*p*
Mean age (years)	46.21±6.14	45.57±6.48	0.106
BMI	23.16±3.92	22.68±3.54	0.062

Table 3. cfDNA levels in peripheral blood of patients, relative to control.

Time	Group (ng/mL)		*p*
	Observation (n = 80)	Control (n = 50)	
Before treatment	15.57±4.80*	8.49±1.89	0.024
7th day of treatment	15.66±3.35*		0.023
20th day of treatment	14.44±2.73$^{*\#}$		0.036

$^{*}p<0.05$ compared with control group; $^{\#}p < 0.05$ compared with 1 day before treatment.

Table 4. Comparison of long fragment DNA plasma levels in breast cancer patients.

Time	Group (ng/mL)		*p*
	Observation (n = 80)	Control (n = 50)	
Before treatment	2.32±1.23*	0.75±0.18	0.016
Seventh day of treatment	2.40±1.32*		0.014
The 20th day of treatment	1.97±1.06*#		0.026

*$p<0.05$ compared with the control group; #$p<0.05$ compared with 1 day before treatment

treatment ($p<0.05$). These results are shown in Table 3.

Comparison of long fragment DNA plasma levels in breast cancer patients and controls

As shown in Table 4, the long fragment DNA level in breast cancer patients before treatment was significantly higher than that of the control group ($p<0.05$). There was no significant difference between fragment DNA value at 7 days after treatment and the value at 1 day before treatment ($p>0.05$). However, fragment DNA decreased significantly on the 20th day of treatment, when compared with the corresponding levels before treatment ($p<0.05$).

Diagnostic value of cfDNA and long fragment DNA in breast cancer patients

Before treatment, the optimal cut-off point for cfDNA in patients peripheral blood, sensitivity, specificity and accuracy were 12.25ng/mL, 79.12%, 86.15%, and 73.32%, respectively. The AUC was 0.865 (95% CI = 0.754-0.903).

Discussion

Circulating free DNA (also known as cell-free DNA, cfDNA) is degraded DNA fragments released to the blood plasma. The term describes various forms of DNA circulating freely in body fluids or blood (12, 13). In normal healthy individuals, cfDNA comes principally from apoptotic cells: only a few arise from necrotic cells. However, in cancer patients, most of the cfDNA is released by necrotic cells (14, 15). The fragments of DNA produced by tumor cell necrosis are diverse in length due to incomplete digestion and consist mainly of long fragment DNA (16, 17). The release of cfDNA into the bloodstream is triggered by factors such as a primary tumor, tumor cells that circulate in peripheral blood, metastatic deposits present at distant sites, and normal cell types such as hematopoietic and stromal cells.

Tumor burden (also called tumor load) refers to the number of cancer cells, the size of a tumor, or the amount of cancer in the body. During the diagnosis and treatment of tumors, dynamic monitoring of changes in tumor load has some clinical significance (18, 19). It has been reported that the levels of cfDNA and long fragment DNA in peripheral blood indirectly reflects changes in the tumor burden, and may be used to assess the biological activity of tumors (20, 21). The present study investigated the diagnostic value of cfDNA and long fragment DNA in breast cancer patients. The results showed that the level of cfDNA in breast cancer patients before treatment was significantly higher than that of the control group. The cfDNA level of patients 7 days after treatment was not significantly different from that at 1 day before treatment but decreased significantly on the 20th day of treatment when compared with the corresponding levels before treatment. These results indicate that the cfDNA level in peripheral blood may reflect the pathological status, and agree with reports of previous studies (22). It could be that the amount of DNA released by breast cancer cells exceeded the maximum level that could be cleared at a particular time (23). Hence, breast cancer patients may be diagnosed early by measuring the cfDNA level in peripheral blood. It has been demonstrated that the cfDNA level in breast cancer patients is positively correlated with the degree of severity of the disease (24). The results of this study also suggest that the severity of a patient's condition and prognosis may be determined using the plasma level of cfDNA (25).

Metastasis, necrosis and apoptosis of tumor cells result in the release of a large amount of long fragment DNA into the peripheral blood. The presence of DNA inhibitors in peripheral blood of patients suppresses DNA degradation, thereby elevating long fragment DNA in plasma of breast cancer patients (26). In this study, long fragment DNA level of patients 7 days after treatment was not significantly different from the corresponding level at 1 day before treatment, but it decreased significantly on the 20th day of treatment when compared with the corresponding levels before treatment. It is likely that on day 7, the patients were in the recovery phase of the disease. The results also suggest that cfDNA and long fragment DNA levels in peripheral blood may not be effective in determining tumor load. However, on the 20th day of treatment, the microenvironment may have gradually stabilized, with minimal interference. At this point, the levels of cfDNA and long fragment DNA in peripheral blood may effectively reflect tumor load (27, 28). Therefore, close monitoring of cfDNA and long fragment DNA levels in peripheral blood of breast cancer patients offers a reliable and early diagnosis for patients. It may also provide a robust assessment for clinical effectiveness and disease prognosis (29). The results obtained in this study indicate that cfDNA may have high diagnostic and prognostic value in breast cancer patients, and are in agreement with reports of previous studies (29). Therefore, cfDNA may serve as a sensitive, specific and accurate tumor marker for breast cancer.

Close monitoring of the cfDNA level in peripheral blood of breast cancer patients in real-time may be used for early diagnosis, treatment and prognosis of the disease.

Acknowledgments
None.

Conflicts of interest
There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Haiyan Xu and Guirong Liu; Haiyan Xu, Guirong Liu collected and analysed the data; Haiyan Xu wrote the text and all authors have read and approved the text before publication. Haiyan Xu and Guirong Liu are co-corresponding authors.

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